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Inhibition of cytokines and JAK–STAT activation by distinct signaling pathways

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ABSTRACT **An important component of cytokine regulation of cell growth and differentiation is rapid transcriptional activation of genes by the JAK–STAT (signal transducer and activator of transcription) signaling pathway. Ligation of cytokine receptors results in tyrosine phosphorylation and activation of receptor-associated Jak protein tyrosine kinases and cytoplasmic STAT transcription factors, which then translocate to the nucleus. We describe the interruption of cytokine triggered JAK–STAT signals by cAMP, the calcium ionophore ionomycin, and granulocyte/macrophage colony-stimulating factor. Jak1 kinase activity, interleukin 6-induced gene activation, Stat3 tyrosine phosphorylation, and DNA-binding were inhibited, as was activation of Jak1 and Stat1 by interferon γ. The kinetics and requirement for new RNA and protein synthesis for inhibition of interleukin 6 by ionomycin and GM-CSF differed, but both agents increased the association of Jak1 with protein tyrosine phosphatase 1D (SH2-containing phosphatase 2). Our results demonstrate that crosstalk with distinct signaling pathways can inhibit JAK–STAT signal transduction, and suggest approaches for modulating cytokine activity during immune responses and inflammatory processes.**

An important advance in understanding the mechanism by which cytokines regulate cells has been the identification and characterization of the JAK–STAT (signal transducer and activator of transcription) signal transduction pathway (1–5). The binding of many cytokines and growth factors to their receptors activates Janus kinases (Jaks), which are protein tyrosine kinases that are physically associated with the receptor. Typically, stimulation by a particular cytokine results in the activation of a distinct pair of two of the four known Jaks. Jak kinases are required for tyrosine phosphorylation and activation of latent cytoplasmic transcription factors termed STATs. STATs are rapidly tyrosine phosphorylated after stimulation with cytokines, and subsequently dimerize and translocate to the nucleus, where they can activate transcription.

Six distinct but homologous members of the STAT family have been identified and designated Stat1 through Stat6 (1–5). Most STATs are widely expressed in a variety of cell types. An individual STAT protein may be activated by multiple ligands, but certain ligands preferentially activate particular STATs. For example, interferon (IFN) γ preferentially activates Stat1 (1), interleukin (IL) 6 preferentially activates Stat3 (6, 7), and IL-4 preferentially activates Stat6 (8). The interaction of STAT SH2 domains with receptor sequences is an important determinant of the specificity of STAT activation (4).

Recent work has shown that regulation of JAK–STAT signaling is more complex than activation of specific STATs in response to ligation of receptors of the cytokine receptor superfamily (4, 9–22). Enhanced STAT activity in cells transformed by human T-lymphotropic virus type I or by v-Src or v-Abl oncoproteins, and after ligation of the G protein-coupled angiotensin II receptor (9–12), suggest that signaling pathways in addition to those triggered by cytokines may be important in activation of STAT DNA-binding. Crosstalk and convergence with different signaling pathways, such as mitogen-activated protein kinase pathways, that regulate serine phosphorylation of STATs may be important for full activation of DNA-binding or transcriptional activity (13–16). Furthermore, cellular differentiation over several days or exposure to immune complexes can result in altered activation of the JAK–STAT pathway and altered cellular responses to cytokines (17–22). We have begun to explore whether cytokine activity can be modulated or inhibited by interrupting signaling through the JAK–STAT pathway. Our results show that several different signaling pathways can interrupt JAK–STAT signaling, likely at a proximal step, by inhibiting Jak1 kinase activity.

MATERIALS AND METHODS

Cell Isolation and Culture. Mononuclear cells (MNC) from disease-free volunteers were obtained from whole blood or buffy coats (New York Blood Center) by density gradient centrifugation using Ficoll metrizoate (Lymphoprep; GIBCO/BRL). Monocytes were purified by depleting T cells using rosetting with sheep red blood cells, followed by negative selection with magnetic beads against remaining T and B cells (Dynal, Great Neck, NY). Monocytes were >80% pure as determined by flow cytometry. MNC or monocytes were cultured at a cell density of $1–2 \times 10^7$ cells/ml in complete medium (RPMI medium 1640 supplemented with 10% fetal bovine serum) and stimulated with agents and cytokines, as detailed in the figure legends.

DNA Binding Assays. Cell extracts were prepared by lysis in buffer containing 20 mM Hepes (pH 7.0), 300 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 0.1% Triton X-100, 0.5 mM DTT, 200 mM phenylmethylsulfonyl fluoride, and 20% glycerol as described (23). The protein concentration of extracts was determined using the Bradford assay (24) and 8 μg of extract was incubated with 0.5 ng of ^{32}P-labeled double-stranded oligonucleotide probe as described (23). In supershift experiments, 1 μl of a 1:10 dilution of specific antiserum (6) was added to extracts for 15 min before adding radiolabeled probe. Samples were resolved on 4.5% polyacrylamide gels in 0.25× TBE buffer at 11 V/cm at room temperature.

Northern Blot Hybridization Analysis. Total cellular RNA was isolated using RNAzol (Cinna/Biotecx Laboratories, Friendswood, TX) according to the instructions of the man-

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Abbreviations: STAT, signal transducer and activator of transcription; Jak, Janus kinase; IL, interleukin; GM-CSF, granulocyte/macrophage colony-stimulating factor; IFN, interferon; hSIE, high affinity serum-inducible element; PTP, protein tyrosine phosphatase; MNC, mononuclear cells; SF, synovial fluid.

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ufacturer. RNA (5 μg) was fractionated on 1.2% formaldehyde agarose gels, transferred to Hybond-N membranes, and hybridized with random primer labeled (Boehringer Mannheim) cDNA probes using standard techniques, as described (23).

Immunoprecipitations and Immunoblotting. Extract volume was adjusted to 500 μl using extraction buffer supplemented with 2 mM sodium orthovanadate, 10 mM NaF, and protease inhibitors (23), 500 μl of H_2O was added, and extract was incubated with 1–2 μl of preimmune serum, 1 μl of Stat3 antiserum (6), 2 μl of Jak 1 antiserum (25), or 4 μg of affinity purified protein tyrosine phosphatase (PTP) 1D antibodies (Santa Cruz Biotechnology) at 4°C for 2 hr. Immunoprecipitates were collected using protein A- and protein G-agarose beads (Pierce), washed three times with 0.5× extraction buffer with phosphatase and protease inhibitors, once with PBS, fractionated on an SDS/7.5% polyacrylamide gel, and transferred to polyvinylidene fluoride membranes (Amersham). Antiphosphotyrosine antibody 4G10 (1 μg/ml; Upstate Biotechnology, Lake Placid, NY) or monoclonal anti-PTP 1D antibody, or a 1:1000 dilution of Stat3 or Jak1 antiserum was used for immunoblotting. Detection was by the Enhanced Chemiluminescence kit (Amersham). Similar results were obtained using different antibodies against the same proteins (Transduction Laboratories, Lexington, KY).

***In Vitro* Kinase Assays.** Cells were lysed in buffer (26) containing 0.5% Nonidet P-40, 10% glycerol, 50 mM Tris·HCl (pH 8.0), 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 1 mM sodium orthovanadate, and protease inhibitors. Preimmune or Jak1 antiserum (2 μl) was used and immunoprecipitates were washed twice with lysis buffer, twice with kinase buffer (26) containing 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM Na_3VO_4, 10 mM Hepes (pH 7.4), resuspended in 40 μl of kinase buffer, and incubated at room temperature for 30 min with 100 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP. Proteins were resolved by SDS/PAGE and ^{32}P-labeled proteins were detected by autoradiography.

RESULTS

Inhibition of IL-6 by Ionomycin, cAMP, and Granulocyte/Macrophage Colony-Stimulating Factor (GM-CSF). We screened a panel of cytokines and compounds for the ability to inhibit activation of STATs by IL-6, a cytokine that activates Stat1 and Stat3, and plays an important role in immune and inflammatory responses (6, 7, 23, 27). Stimulation of freshly isolated blood MNC with inflammatory synovial fluid (SF) (23) or purified recombinant IL-6 resulted in the induction of protein complexes that bound to the high affinity serum-inducible element (hSIE) oligonucleotide (6) that preferentially binds Stat1 and Stat3 (Fig. 1*A*, lanes 2 and 9). These complexes reacted specifically with Stat1 and Stat3 antisera (6) (lanes 3 and 4), and we have previously shown that SF-induction of Stat1 and Stat3 is mediated solely by IL-6 (23). Thus, IL-6 activated both Stat1 and Stat3 in mononuclear blood cells, and subsequent experiments showed that IL-6 activated predominantly Stat3 in purified monocytes, and Stat1 in purified T cells (data not shown). Of greater than 20 agents tested for inhibitory activity, three agents, cAMP, the calcium ionophore ionomycin, and GM-CSF, inhibited IL-6-induced binding of Stat1 and Stat3 (Fig. 1*A*, lanes 5–7, 10, and 11; a representative experiment from over 20 different blood donors tested is shown).

DNA-binding activity of STATs depends primarily upon tyrosine phosphorylation (1–5), although serine phosphorylation may be important in modulating binding affinity of Stat3 (13). We analyzed tyrosine phosphorylation of Stat3 using immunoprecipitation from cell lysates, followed by phosphotyrosine immunoblotting (Fig. 1*B*). Tyrosine phosphorylation of Stat3, which was induced by IL-6 (lane 2) or by SF (lane 5), was completely inhibited by ionomycin and partially inhibited by GM-CSF (lanes 3, 4, and 6). The extent of inhibition of tyrosine phosphorylation correlated with the extent of inhibition of DNA-binding in the same experiment (Fig. 1*A*, lanes 9–11). Reprobing the filters with Stat3 antiserum demonstrated comparable levels of immunoprecipitated Stat3 in all lanes (Fig. 1*B Lower*). Thus, inhibition of Stat3 DNA-binding activity was achieved primarily through inhibition of tyrosine phosphorylation. Analysis of IL-6 receptor expression showed that inhibition was not secondary to down-regulation of IL-6 receptors (data not shown).

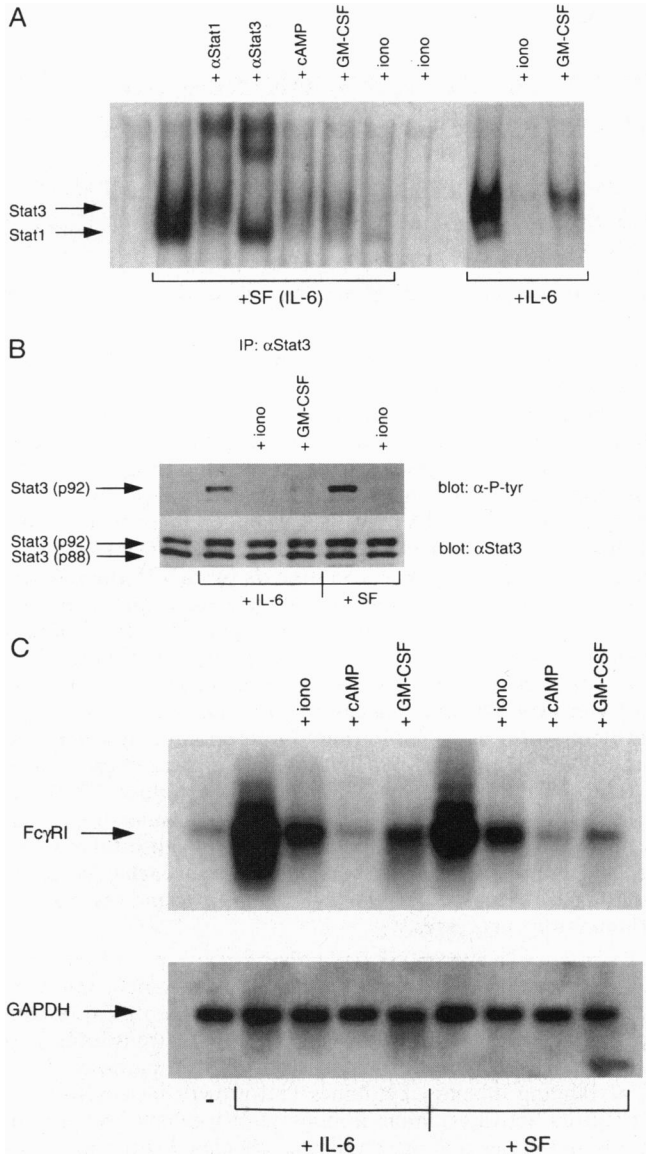

FIG. 1. Inhibition of IL-6 activation of STATs and FcγRI expression. (*A*). Freshly isolated blood MNC were incubated for 1 hr in complete medium with 1 mM 8-Br-cAMP, 200 unit/ml GM-CSF, or 1 μg/ml ionomycin, followed by a 15-min exposure to SF (containing IL-6) or 20 ng/ml of IL-6. Cell extract (8 μg) was assayed for binding to a radiolabeled hSIE oligonucleotide (containing a STAT binding site) using gel shift assays. In lanes 3 and 4, 1 μl of a 1:10 dilution of specific anti-Stat1 or anti-Stat3 antiserum (6) was incubated with extracts for 15 min before adding radiolabeled probe. (*B*) Extracts obtained from 10^8 MNC were immunoprecipitated with Stat3 antiserum and immunoprecipitates were analyzed using immunoblotting with antiphosphotyrosine antibodies. Membranes were stripped and reprobed with Stat3 antiserum. (*C*) Cells were harvested 6 hr after addition of IL-6, and 5 μg of total cellular RNA was fractionated on an agarose formaldehyde gel, transferred to a nylon membrane, and hybridized with radiolabeled cDNA probes.

The effect of inhibiting Stat1 and Stat3 activation upon the expression of an IL-6- and SF-inducible gene, FcγRI, was investigated. cAMP, ionomycin, and GM-CSF inhibited the induction of FcγRI mRNA levels by IL-6 or SFs (Fig. 1*C*) and also inhibited induction of cell surface expression of FcγRI (data not shown). Thus, these agents block IL-6 activity and have functional consequences for cellular responses to this cytokine. Since the FcγRI gene is primarily transcriptionally regulated and its promoter contains a sequence that binds Stat1 and Stat3 and is important for transcriptional activation (23, 28), the block in STAT activation likely contributes to the inhibition of induction of FcγRI mRNA levels. However, the strong inhibition of FcγRI mRNA expression by cAMP, which only partially blocked STAT activation, suggests that additional pathways may be inhibited. These results show that at least two distinct major cellular signaling pathways that can inhibit immune responses (29–32), those involving the serine protein kinases, calmodulin kinase, and protein kinase A, can inhibit STAT DNA-binding activity, and inhibition has important functional consequences for the expression of IL-6 regulated genes.

Inhibition of Jak1 Kinase Activity and Activation of Stat1. The effect of ionomycin and GM-CSF on components of the IL-6 signaling pathway was examined. Monocytes were used because pilot experiments showed that monocytes were more susceptible to inhibition than other cell types contained in MNC and cell lines (data not shown). Jak kinases are associated with cytokine receptors (1–5), and activation of Jak1 is necessary for IL-6 signal transduction and activation of STATs (26). Immunoprecipitation and *in vitro* kinase assays revealed Jak1 kinase activity that was detected only when specific anti-Jak1 antiserum (25) was used, was present at baseline after culture in complete medium (containing serum growth factors), and increased minimally after stimulation with 20 ng/ml of IL-6 (Fig. 2*A Upper*, lanes 1–3). Jak1 kinase activity corresponded to Jak1 tyrosine phosphorylation detected after immunoprecipitation with several anti-Jak1 antibodies (data not shown). Treatment with ionomycin and GM-CSF resulted in decreased Jak1 kinase activity (Fig. 2*A*). Comparable levels of immunoprecipitated Jak1 were verified using immunoblotting with a fraction of immunoprecipitated samples (Fig. 2*A Lower*).

In contrast to treatment with 20 ng/ml of IL-6 (the minimal dose required to fully activate Stat3), treatment with 20 ng/ml (500 units/ml) of IFN-γ induced a robust increase in Jak1 kinase activity (Fig. 2*B Middle*). IFN-γ-induced kinase activity was inhibited by ionomycin and GM-CSF, but residual kinase activity remained, and Stat1 activation by IFN-γ was only minimally inhibited (Fig. 2*B Top*). Dose response experiments showed that activation of Stat1 DNA-binding by IFN-γ was near maximal at doses as low as 1 unit/ml (data not shown). Fig. 2*C* shows that addition of 1 unit/ml of IFN-γ resulted in Stat1 activation, but no increase in Jak1 kinase activity was detected, even when baseline kinase activity was low in cells cultured in the absence of serum. Higher doses of IFN-γ minimally increased levels of DNA-binding, despite a substantial induction of Jak1 kinase activity (Fig. 2*C*); the apparent relatively low level of activation of Jak1 by 500 unit/ml of IFN-γ was secondary to inefficient immunoprecipitation (Fig. 2*C Bottom*). Activation of Stat1 by low doses of IFN-γ was inhibited by ionomycin (Fig. 2*D*).

These data show that ionomycin and GM-CSF inhibit Jak1 kinase, a proximal molecule in the signaling pathways of IL-6, IFN-γ, and many cytokines, and suggest that a near complete block in kinase activity is required to block activation of Stat1 and Stat3. An important role for Jak1 expression and kinase activity in the activation of Stat1 and Stat3 has been demonstrated using a genetic approach with cells that do not express Jak1 or express a kinase-deficient Jak1 mutant (1, 26, 33). Activation of Stat3 by IL-6 and Stat1 by IFN-γ occurred with relatively low levels of Jak1 kinase activity. Higher levels of Jak1 activation require high doses of cytokine, such as 400 ng/ml IL-6 plus 500 ng/ml of soluble IL-6 receptor (26), and

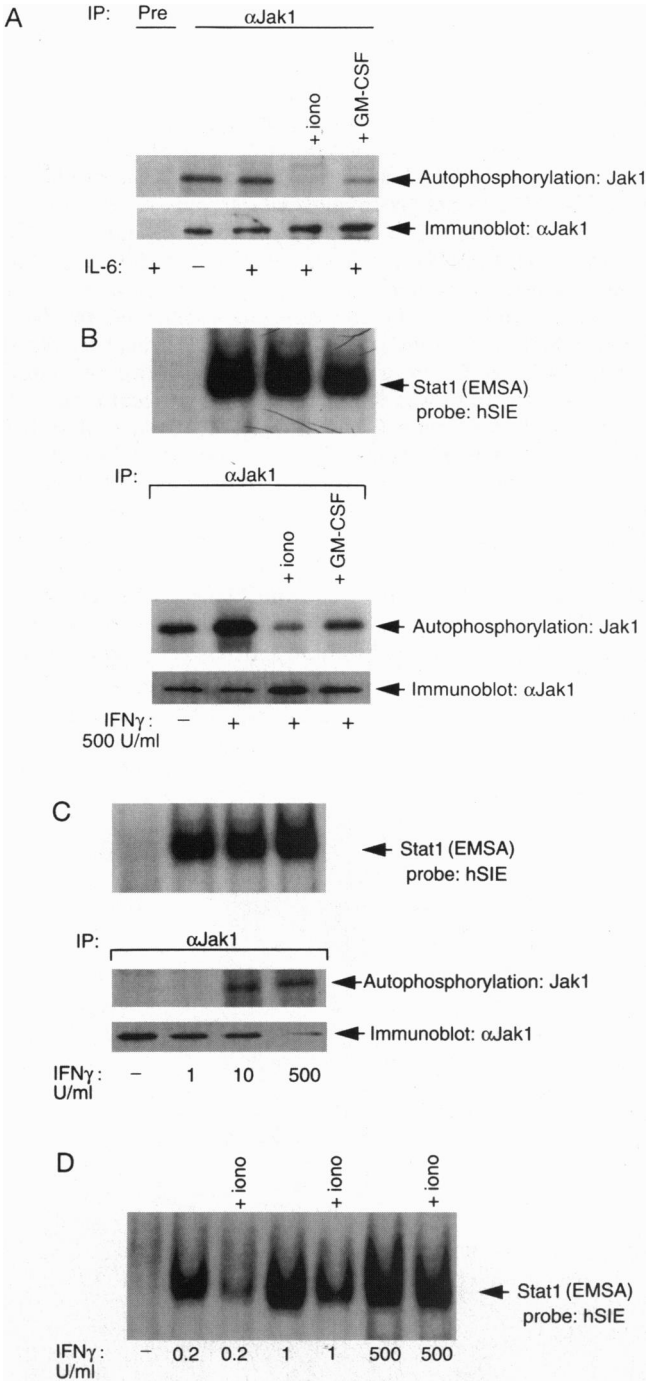

FIG. 2. Regulation of Jak1 kinase activity by ionomycin and GM-CSF. (*A*) Monocytes were incubated for 1 hr in complete medium with 1 μg/ml ionomycin or 200 unit/ml GM-CSF, followed by a 15-min stimulation with 20 ng/ml of IL-6. Extracts from 2×10^7 monocytes were immunoprecipitated with preimmune or Jak1 antiserum (25), followed by an *in vitro* kinase assay and analysis of Jak1 autophosphorylation. A fraction of the immunoprecipitate was analyzed using immunoblotting with Jak1 antiserum (*Lower*). (*B*) IFN-γ (500 units/ml; 20 ng/ml) was used and extracts were assayed for binding to the hSIE oligonucleotide before immunoprecipitation and kinase assay. (*C*) Monocytes were treated with IFN-γ for 10 min in serum-free medium and cell extracts were assayed for DNA binding, followed by immunoprecipitation and kinase assay. (*D*) Monocytes were treated as in *A* and extracts assayed for binding to the hSIE oligonucleotide.

may be required for activation of additional signaling pathways, such as those leading to an antiviral state or cellular proliferation, by Jak1 (5, 33). Inhibition of various signaling pathways activated by Jak1 will likely be determined by the balance of Jak1 activation by a particular cytokine and the extent of inhibition by ionomycin and GM-CSF.

Requirement of RNA and Protein Synthesis for Inhibition by GM-CSF but Not by Ionomycin. The possibility that the mechanism of inhibition of IL-6 by ionomycin and GM-CSF involved crosstalk at the level of signal transduction was analyzed in greater detail. We reasoned that if the JAK–STAT pathway is inhibited directly by signals triggered by these agents, inhibition should occur after a short preincubation and should not require prior synthesis of a negative regulator. Activation of Stat3 was inhibited by a 5-min treatment with ionomycin but not with GM-CSF [Fig. 3*A*; a dose of IL-6 5 (ng/ml) that does not activate Stat1 was used]. GM-CSF did activate a STAT protein that bound only to the IRF oligonucleotide (34) (Fig. 3*A*), and likely represents Stat5 (2–5). Preincubation with actinomycin D and cycloheximide, inhibitors of new RNA and protein synthesis, respectively, did not affect the activity of ionomycin, but blocked the activity of GM-CSF (Fig. 3*B*). These results suggest that a calcium-triggered signal can directly inhibit JAK–STAT activation, but

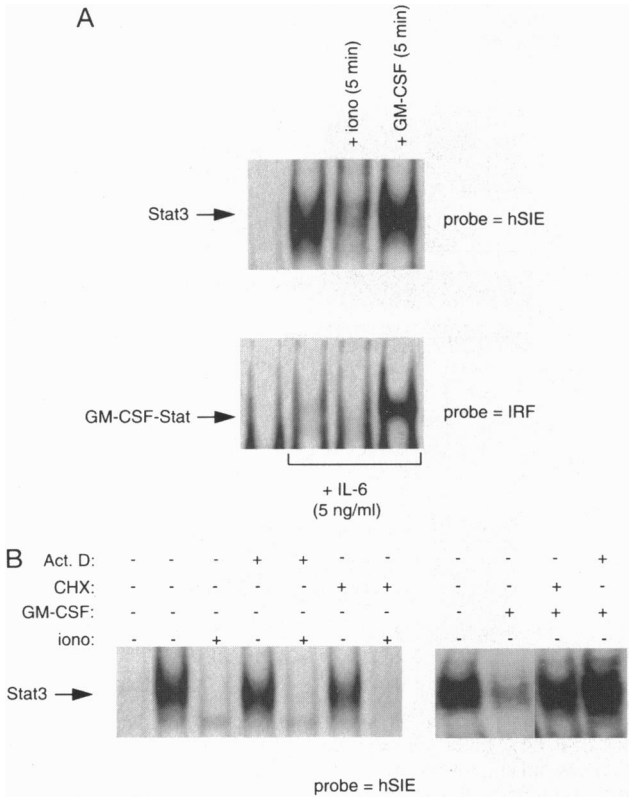

FIG. 3. Inhibition of IL-6 by ionomycin, but not by GM-CSF, is rapid and independent of new RNA and protein synthesis. (*A*) MNC were treated with inhibitors for 5 min before a 15-min stimulation with 5 ng/ml of IL-6 (a dose that activates only Stat3). Cell extracts were assayed for binding to radiolabeled hSIE or IRF oligonucleotides under binding conditions where binding of Stat3 to the IRF oligonucleotide was not detected (23, 34). (*B*) Cells were incubated for 15 min in the presence or absence of 5 μg/ml actinomycin D or 20 μg/ml cycloheximide; ionomycin (1 μg/ml) or GM-CSF (200 unit/ml) was added for 1 hr, followed by a 15-min stimulation with IL-6. In lanes 2–7, MNC were stimulated with 5 ng/ml of IL-6, and in lanes 8–11, monocytes (in which IL-6 activates predominantly Stat3) were stimulated with 20 ng/ml of IL-6. DNA binding activity was assayed using the hSIE oligonucleotide.

the GM-CSF effect depends upon induction of a factor that contributes to inhibition.

Regulation of Association of Jak1 and PTP 1D by Inhibitors. PTP 1D (also termed SH-PTP2, Syp, or SHP2) interacts with the gp130 signaling component of the IL-6 receptor (35, 36), and also may associate with Jak kinases independently of gp130, possibly through interactions with Grb2 (36, 37). PTP 1D/SHP2 may transmit both positive and negative signals (3, 38–43), and inhibition of signaling by PTP 1D would be similar to negative regulation of erythropoietin and IFNα signaling by the related phosphatase PTP 1C (SHP1) (44, 45). The interaction of PTP 1D with Jak1 was investigated using coimmunoprecipitation assays. Ionomycin and GM-CSF treatment resulted in increased coimmunoprecipitation of PTP 1D with Jak1 (Fig. 4*A*), and, in a reciprocal experiment, in increased Jak1 coimmunoprecipitation with PTP 1D (Fig. 4*B*). Increased coimmunoprecipitation was not detected after cAMP treatment (data not shown), consistent with a different proposed mechanism of inhibition by cAMP (46). Immunoprecipitation of Jak1 or PTP 1D was not observed with irrelevant antibodies (Fig. 4 *A* and *B*), and coimmunoprecipitation was increased only under stimulation conditions that inhibited binding: 1-hr incubation with ionomycin or GM-CSF or 5min incubation with ionomycin, but not after a 5-min stimulation with GM-CSF (Fig. 4*B*, lane 7), that also did not inhibit binding activity (see Fig. 3*A*). These observations were confirmed using different antibodies to Jak1 and PTP 1D, and coimmunoprecipitating gp130 was not detected (data not shown). Furthermore, increased coimmunoprecipitation of Jak1 with PTP 1D after GM-CSF treatment (Fig. 4*C Lower Left*, lanes 1 and 2; the lower band in lane 2 corresponds to Jak1 and comigrated with Jak1 protein in the control extract shown in lane 4) was blocked by actinomycin D (Fig. 4*C*, lane 3), which also blocked GM-CSF inhibition of Stat3 DNA-binding (Figs. 3*B* and 4*C Right*). The use of purified monocytes (2×10^7 cells per immunoprecipitation) instead of MNC (10×10^7 cells per immunoprecipitation) led to weak Jak1 signals relative to a more slowly migrating band (Fig. 4*C*), whose levels were not regulated by GM-CSF or actinomycin D, and whose identity is not currently known. These results provide further evidence for the regulation of proximal signaling events by ionomycin and GM-CSF, and suggest that PTP 1D may mediate inhibition by dephosphorylating and inactivating Jak1. This is similar to inhibition of Jak kinases by PTP 1C (44, 45), except that PTP 1D appears to be regulated by additional extracellular signals.

DISCUSSION

An important role is emerging for convergence and crosstalk between signaling pathways in the activation of STAT proteins (4, 9–22). Our results show that crosstalk can also result in inhibition of JAK–STAT signaling and cytokine activity. JAK–STAT signaling was blocked by an antagonistic cytokine and by signal transduction pathways that activate serine/threonine kinases and are typically triggered by noncytokine ligands. The mechanism involved inhibition of tyrosine phosphorylation of STATs and of Jak1 kinase activity. Interestingly, tyrosine phosphorylation mediated by the insulin receptor, which contains intrinsic kinase activity, is also inhibited by signals that trigger serine phosphorylation, specifically of the IRS-1 molecule (47). IRS-1 is not known to participate in IL-6 or IFN-γ signaling, but does play a role in signaling by IL-4, which is also inhibited by ionomycin (T.K.S., unpublished data). The substrates of serine/threonine kinases that mediate inhibition of IL-6- and IFN-γ-triggered JAK–STAT signaling remain to be defined. Possible substrates include receptor chains, such as gp130, and phosphatases, such as PTP 1D, both of which can be modified by serine/threonine phosphorylation (39, 48).

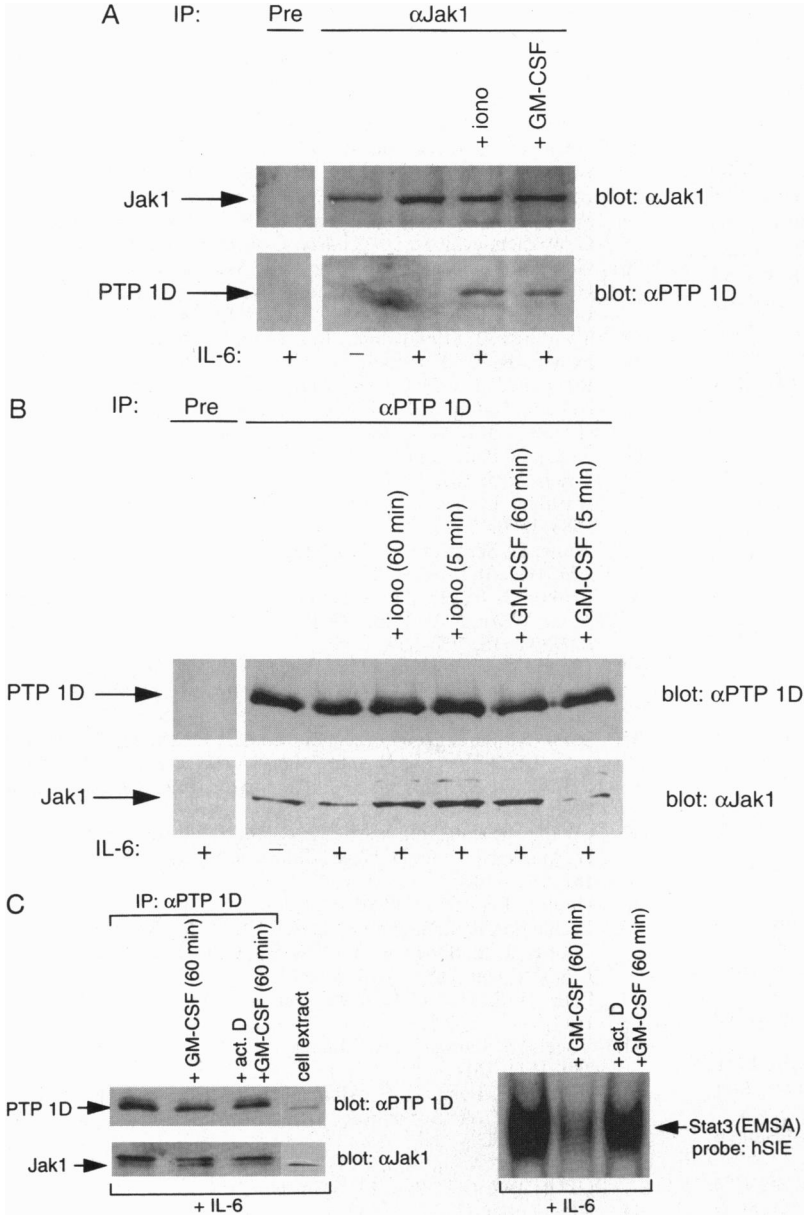

FIG. 4. Ionomycin and GM-CSF induce an increased association of Jak1 with PTP 1D. (*A* and *B*) MNC were pretreated with 1 μg/ml ionomycin or 200 unit/ml GM-CSF for 1 hr before a 15-min stimulation with 20 ng/ml of IL-6. Extracts from 10^8 MNC were immunoprecipitated with preimmune, Jak1 (*A*), or PTP 1D (*B*) antisera, and immunoprecipitates were analyzed using immunoblotting with the indicated antibodies. (*C*) Purified monocytes were used, and extracts from 2×10^7 monocytes were immunoprecipitated with PTP 1D antiserum. Lane 4 contains a control extract to provide a marker for Jak1, and a shorter exposure of the same filter is shown. (*Right*) A DNA-binding assay using the same extracts as in lanes 1–3 at *Left*, but before immunoprecipitation.

Treatment with ionomycin and GM-CSF resulted in inhibition of Jak1 kinase, a proximal kinase in signaling pathways activated by IL-6, IFN-γ, and additional cytokines (1–5). Given the strength of the genetic evidence linking activation of Jak1 and activation of STATs by IL-6 and IFN-γ (26, 33), it is likely that STAT activation was prevented by a block upstream in the signaling pathway, namely, inhibition of Jak1, rather than increased dephosphorylation or turnover of activated STAT molecules. Our data suggest that only low levels of Jak1 kinase activity are necessary for activation of STAT DNA-binding activity, and that kinase activity must be nearly completely blocked to prevent activation of STATs. The apparent low levels of Jak1 activity after stimulation with IL-6 and low doses of IFN-γ (Fig. 2) are likely secondary to activation of only a small fraction of total cellular Jak1 kinases after ligation of IL-6 receptors (expressed at low levels) or after ligation of a small fraction of IFN-γ receptors by doses of IFN-γ in the 0.2–1.0 unit/ml range.

Based upon the regulated interaction of PTP 1D and Jak1 and the reported activation of PTP 1D phosphatase activity after binding to a substrate (39), it is plausible to propose that PTP 1D may participate in inhibition of JAK–STAT signaling, possibly by dephosphorylating and inactivating Jak1. The several-fold increased association of PTP 1D with Jak1, if targeted to specific receptors, could account for the block in STAT phosphorylation, especially if potential activation of phosphatase activity (up to 10-fold has been reported; ref. 39) is taken into account. However, PTP 1D also has positive signaling functions (38), and further work will be required to delineate the exact role of PTP 1D in JAK–STAT signaling. The increased association of PTP 1D with Jak1 after treatment with ionomycin and GM-CSF provides further support for the hypothesis that these agents affect early and proximal steps in JAK–STAT signal transduction, before activation of STATs. PTP 1D has been reported to interact with a number of receptors, cytoplasmic molecules, and adaptor molecules (35, 36, 38–43), and it is possible that the Jak1–PTP 1D association is mediated by intermediary molecule(s) or adaptor(s). Ionomycin and GM-CSF may function by inducing, respectively, posttranslational modification or expression, of adaptor molecules that then associate with PTP 1D and Jak1. One obvious candidate for an adaptor molecule, gp130, has not been detected in coimmunoprecipitation experiments to date (T.K.S., unpublished data). Another intriguing possible can-

didate would be a molecule similar to or related to IRS-1, which associates with both PTP 1D and Jak1 (49), plays a role in signaling by insulin and IL-4, is regulated by serine phosphorylation, and inhibits insulin-triggered tyrosine kinase activity (47).

In a physiologic setting where cells are exposed to numerous stimuli simultaneously, transcriptional responses to cytokines will be modulated by an interplay among synergistic and antagonistic signals. Our results reveal one mechanism by which cytokine signaling may be modified by different stimuli or antagonistic cytokines through a block in the JAK–STAT signaling pathway. Inhibition of cytokine-triggered transcriptional responses likely plays an important role in modulation of immune and inflammatory responses, and pharmacologic blockade of JAK–STAT signaling may represent an effective approach to therapy of inflammatory diseases.

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